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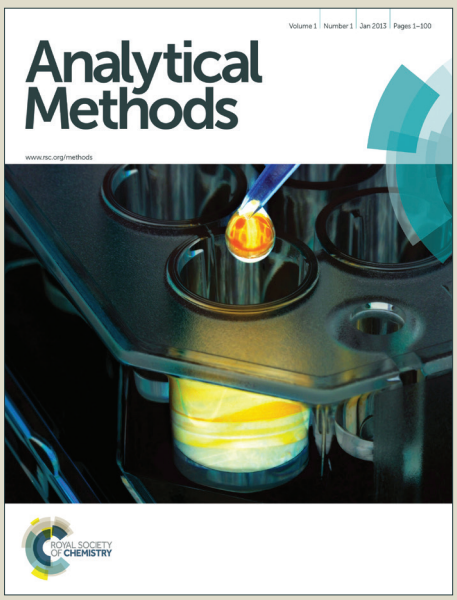

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ARTICLE TYPE

Label-free fluorescent assay of ATP based on aptamer-assisted light-up of Hoechst dyes

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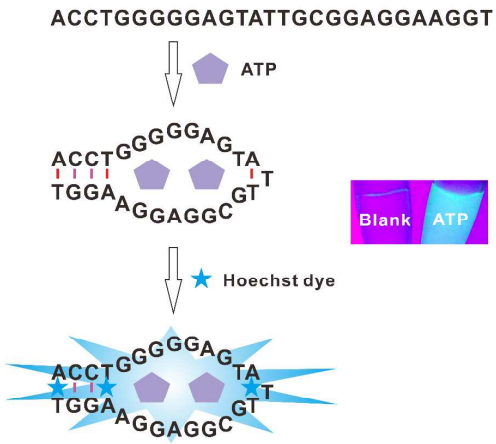
A label-free fluorescent assay was developed for light-up detection of ATP in a visual-readout format based on the aptamer-directing fluorescence of Hoechst dyes.

Hoechst dyes are a group of blue fluorescent dyes served as cell permeable nucleic acid stains,[1] which are AT-selective minor groove binding dyes. The fluorescence of Hoechst dyes can be enhanced considerably upon binding to AT-rich double-stranded DNA (dsDNA).[2] Some label-free probes have been developed based on this sequence-dependent fluorescence-enhancing property, where certain designed functional DNA sequences and their target molecules were incorporated.[3] Zhu et al. developed a label-free aptamer-based sensor for the detection of L-argininamide by using Hoechst 33258,[3a] Sarpong et al. demonstrated nucleic-acid-binding dyes (eg. Hoechst 33258, etc) can act as efficient indicators of aptamer-target interactions,[3b] and Zhou et al devised an optical aptamer sensor for cocaine using minor groove binder (Hoechst 33342) based energy transfer.[3c] Aptamers are artifical functional nucleic acids (DNA or RNA) that isolated from through *in vitro* selection process or systematic evolution of ligands by exponential enrichment (SELEX) to specifically bind cells, proteins or low-molecular-weight inorganic or organic substrates.[4] Because of their specificity and good binding constants, aptamers have been used in the development of various assay for detection of small molecules, heavy metals, proteins, cancer cells etc.[5]

Recently, our group revealed a designed AT-rich ssDNA can provide a media for enhancing fluorescence of Hoechst dyes to label-free detection of Hg^{2+},[3d] which is based on Hg^{2+}-induced T-Hg^{2+}-T complex[6] to direct the designed AT-rich ssDNA to form a step-loop hairpin; moreover, a novel graphene oxide (GO)-based fluorescent "on/off" switch was developed to visually "turn on" detection of sequence-specific DNA and "turn off" detection of exonuclease with sensitivity and selectivity in a single step in homogeneous solution, utilizing "molecular beacon"-hosted Hoechst dyes (HMB) as signal indicators and GO as an excellent energy acceptor to efficiently quench the fluorescence of HMB in a label-free format.[7]

We describe herein our ongoing effort to develop a facile and label-free assay for ATP, in which aptamer-assisted light-up of Hoechst dyes act as signal indicators (Scheme 1). As shown in Scheme 1, the ATP aptamer can assemble a hairpin-like structure upon challenged with ATP (i.e. target-dependent adaptability of aptamers). ATP-directed formation of a hairpin-like structure (dsDNA with certain A-T basepairing) provides a harbor to accommodate Hoechst dyes as a signal moiety. The free Hoechst displays weak fluorescence, but underwent a marked fluorescence enhancement upon binding to the hairpin-like probe with certain A-T basepairing resulting from the ATP-induced conformational alteration of its aptamer (Apt). In this case, the Apt-Hoechst solution can be used as a selective indicator for ATP.

As shown in Figure 1, the as-prepared Apt-Hoechst solution exhibit fluorescence emission at 506 nm with excitation at 360 nm, however, in the presence of ATP, the fluorescence of the Apt-Hoechst was found to be enhanced by ATP, which is attributed to the ATP-mediated conformational alteration of its aptamer resulting in its self-folding to facilate the harboring of Hoechst dyes into the resultant A-T basepairing, leading to its fluorescence enhance considerably. Considering the appreciable changes in fluorescent enhancement of Apt-Hoechst solution toward ATP, the potential of developing a novel fluorescent assay for determination of ATP was assessed.



Scheme 1. Schematic representation of the use of target-aptamer recognition to regulate fluorescence of Hoechst dyes and its application for light-on detection of ATP.

The different concentrations (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 7.5, 10.0 and 20.0 mM) of

ATP from one stock solution were added to the Apt-Hoechst solution. A gradual increase in the fluorescent intensity (FI) was clearly detected with the addition of an increasing concentration of ATP to the Apt-Hoechst solution (Figure 1A). The ATP-stimulated FI increasement of the Apt-Hoechst solution was rapid. In the presence of 2 mM ATP, the assay exhibited a nearly saturated signal within 2 min (Figure S1), which is faster than previous report using a silver nanoclusters-based label-free fluorescence probe.[8] It can be seen that the FI value is sensitive to the concentraion of ATP, the fitting range is from 0 to 20 mM with a Boltzmann sigmoidal equation ($Y = -5.276 + 14.623/[1 + \exp(1.940 - X)/3.254]$), regression coefficient $R^2 = 0.997$, where Y is the FI ratio at 506 nm and X is the concentration of ATP), additionally, a liner equation can be obtained from the concentration range of 0 to 5 mM ($Y=1.106X + 0.102$, $R^2=0.993$) (Figure 1B). Using the Apt-Hoechst solution, ATP could be detected at concentrations as low as 0.05 mM based on three times signal-to-noise level of the blank sample (3σ). This detection limit was better than that of previous report using a silver nanoclusters-based label-free fluorescence probe.[8]

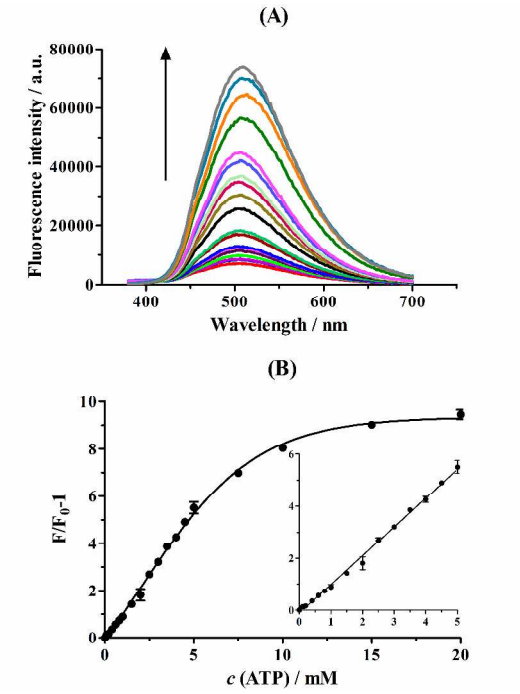


Fig. 1 Fluorescence response of Apt-Hoechst solution to ATP. (A) The fluorescence emission spectra are shown for various ATP concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 7.5, 10.0 and 20.0 mM; (B) The plot of the fluorescence ratio (F/F_0-1) *vs*. the increasing concentrations of ATP of the same data.

To test selectivity, competing stimuli including CTP, GTP and UTP at different molar level were examined under the same conditions as in the case of ATP (Figure 2A). It was found that ATP results in an obvious change in the fluorescence of Apt-Hoechst solution, while there was nearly negligible fluorescent change upon in the presence of other stimuli. Moreover, as shown in Figure 2B, upon addition of ATP and competing stimuli including CTP, GTP and UTP to the Apt-Hoechst solution, a visual readout can be obtained under the 365 nm UV lamp excitation. These results were further confirmed by fluorescence spectroscopy (Figure S2). The results demonstrated the excellent selectivity of this approach applied in ATP detection over competing stimuli. Since there are many artifical functional nucleic acids (eg. aptamers) that reported to selectively bind with a wealth of targets, it can be envisioned that the purposed method in our work may have the potential to be applied widely, if AT-rich region can be found or introduced into the sequence of functional nucleic acids.

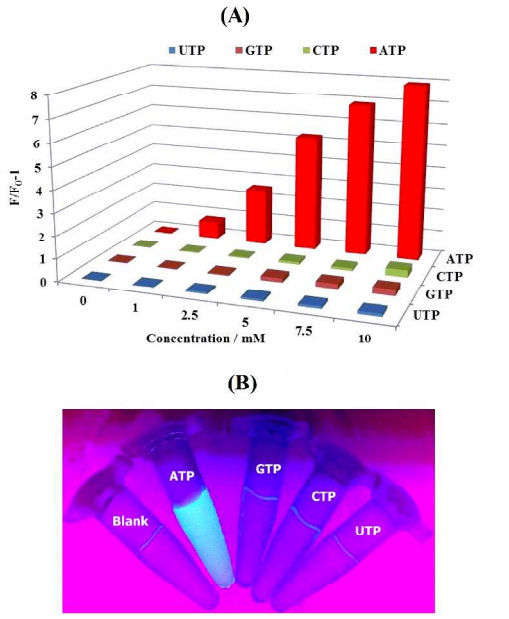


Fig. 2 Selectivity analysis for ATP detection. (A) Bars represent the fluorescence ratio (F/F_0-1) of Apt-Hoechst solution for ATP, CTP, GTP and UTP with concentrations of 0, 1.0, 2.5, 5.0, 7.5 and 10.0 mM, respectively. (B) Visual discrimination of ATP, GTP, CTP and UTP (5.0 mM ATP and 10 mM competing stimuli) based on our proposed method. The picture was taken under the 365 nm handheld UV lamp excitation using a digital camera.

As the evidence now seems strong that ATP is released in large amouts from urothelial cells in response to distention, stress, and inflammation.[9] Urinary ATP quantification can be an important issue in the diagnosis of urinary tract diseases. Therefore, we also studied the possible applicability of the sensor for the direct measurement of ATP in real samples. Under the experimental condition, the proposed method was applied to analyze ATP in artificial urine samples. The results are listed in Table 1. The recovery of the added known amounts of ATP to the 50% artificial urine samples measured by the sensor was in general larger 95%, which indicated that the present method is promising in practical application with great accuracy and reliability.

Table 1. Determination of ATP in real samples using the Apt-Hoechst solution.

Detected (mM)	Added (mM)	Found (mM)	Recovery (%)
Not found	2.00	1.91	95.5
Not found	2.50	2.47	98.8
Not found	3.00	2.97	99.0

In conclusion, we have successfully demonstrated that aptamer-directing fluorescence of Hoechst dyes can be used as a label-free signal indicator for inexpensive, simple, rapid, and selective detection of ATP. This novel "mix and measure" label-free assay design offers many advantages, including simplicity of preparation and manipulation compared with other methods that employ specific strategies including tedious procedures, and the need for labels, etc.

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